

Mechanistic analysis of experimental food allergen-induced cutaneous reactions

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Abstract: Individuals with food allergy often present with urticaria and atopic dermatitis. Indeed, susceptibility to food allergy may predispose to the development of these cutaneous allergic disorders. Recently, we developed a model of food allergy, whereby oral consumption of food [pea *Pisum sativum* L.; expressing α -amylase inhibitor-1 (α AI) from the common bean *Phaseolus vulgaris* L. cv Tendergreen (pea- α AI)] promotes a T helper cell type 2 (Th₂) inflammatory response and predisposes to cutaneous allergic reactions following subsequent food allergen (α AI) exposure. To delineate the kinetics of food allergen-induced cutaneous reactions and examine the inflammatory mechanisms involved in this allergic reaction, we used interleukin (IL)-13-, IL-4 receptor α -, and eotaxin-1-deficient mice and performed serum transfer and CD4⁺ T cell depletion studies. We demonstrate that consumption of pea- α AI promotes an α AI-specific immunoglobulin G₁ (IgG₁) and IgE antibody response. Furthermore, we show that subsequent food allergen (α AI) challenge in the skin induced an early (3 h)- and late-phase (24 h) cutaneous allergic reaction. The early-phase response was associated with mast cell degranulation and the presence of Ig, whereas the late-phase response was characterized by a lymphoid and eosinophilic infiltrate, which was critically regulated by CD4⁺ T cells, IL-13, and eotaxin-1. Collectively, these studies demonstrate that food allergy can predispose to cutaneous inflammatory reactions, and these processes are critically regulated by Th₂ immune factors. *J. Leukoc. Biol.* 80: 258–266; 2006.

Key Words: eosinophil · animal models · food allergy

INTRODUCTION

Atopic dermatitis (AD) is a chronic, inflammatory skin disease characterized by highly pruritic skin lesions [1]. The underlying pathophysiologic mechanisms leading to these clinical manifestations are not yet fully elucidated; however, clinical

studies suggest the involvement of CD4⁺ T cells and eosinophils in disease pathogenesis [1]. Indeed, AD eczematous skin lesions are characterized by a marked perivascular infiltrate consisting of CD4⁺ T cells, and characterization of the cytokine profile has demonstrated increased expression of T helper cell type 2 (Th₂) cytokines, interleukin (IL)-5, and IL-13 as compared with normal subjects. Levels of eosinophils are often elevated in skin lesions of patients with AD, and the presence of this cell correlates with disease activity [2, 3]. In addition, histological evidence of eosinophil degranulation and release of granule proteins, eosinophil cationic protein, eosinophil-derived neurotoxin, and myelin basic protein, have been reported in AD [2]. Consistent with these clinical observations, employing a murine model of AD, investigators have demonstrated a link between Th₂ cells and eosinophils in the pathogenesis of AD [4].

There has been a long-standing debate as to the contribution of diet and food allergies to the clinical manifestations of a variety of cutaneous diseases including acute urticaria and AD [5–7]. Epidemiological analysis has revealed that 8% of asthmatic subjects and 30% of pediatric patients with AD have clinically relevant food allergy [8, 9]. Furthermore, in a double-blind, placebo-controlled food challenge study, investigators demonstrated a positive, late eczematous reaction in 46% of AD children [10]. Consistent with these observations, elimination diets significantly reduce specific immunoglobulin E (IgE) antibodies, peripheral blood mononuclear cell proliferative responses, and symptoms of AD in patients who also suffer from food allergies [11, 12].

Recently, we have developed a model of food allergy that is independent of adjuvant to examine how food allergy sensitization may predispose to and exacerbate cutaneous allergic inflammation [13]. Oral consumption of peas (*Pisum sativum* L.) expressing a gene for α -amylase inhibitor-1 (α AI) promotes a food-allergic α AI-specific CD4⁺ T cell response [13]. Food allergen (α AI) challenge of the skin of mice orally exposed to

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pea- α AI promoted a cutaneous allergic reaction [13]. In the present study, we have used this model to dissect the contribution of Ig, Th₂ cells, and their cytokines in food allergen-induced cutaneous allergic inflammation. We show that oral consumption of pea- α AI predisposes to early (3 h)- and late (24 h)-phase cutaneous inflammatory responses (ECR and LCR, respectively). We show that the ECR was associated with mast cell degranulation and is mediated by Ig, whereas the LCR was associated with a cellular infiltrate consisting primarily of eosinophils. Furthermore, we show that the LCR was dependent on CD4⁺ T cells and involves pathways that employ eotaxin-1, IL-13, and IL-4R α chains. Collectively, these studies suggest that food allergen sensitization can contribute to cutaneous (late eczematous reactions) allergic reactions.

MATERIALS AND METHODS

Mice

Wild-type (WT) BALB/c, eotaxin-1 BALB/c *tm1^{-/-}*, IL-4R α chain BALB/c *tm1^{-/-}* (a gift from Dr. Nancy Noben-Trauth, National Institutes of Health, Bethesda, MD), and IL-13 N5 BALB/c *tm1^{-/-}* mice were obtained from specific pathogen-free facilities at the Australian National University (ANU; Canberra) and housed in approved containment facilities. Mice were treated according to ANU animal welfare guidelines, and age- and sex-matched animals were used throughout these studies.

Intragastric administration of pea and pea- α AI

Pea and pea- α AI were generated as described previously [14]. Plants were supplied, prepared, and administered as described previously [13]. In brief, coarse flour was ground further, homogenized in phosphate-buffered saline (PBS; 0.166 g/mL), and sieved through a 70- μ m mesh. The plant material homogenate was then stored at -20°C until administered. BALB/c-WT, -IL-4R α chain^{-/-}, -IL-13^{-/-}, and -eotaxin-1^{-/-}, knockout (KO) mice were intragastrically (i.g.)-administered 250 μ L pea or pea- α AI plants twice a week for 4 consecutive weeks.

Measurement of hypersensitivity responses in the footpad

One week following i.g. administration of pea or pea- α AI, hypersensitivity responses were determined in the hind footpad by measuring footpad edema as described previously [13]. Hind footpads were injected with 50 μ L α AI (1 mg/mL) or PBS, and hind footpad thickness was measured at 0, 3, 12, 24, and 48 h following challenge using a digmatic calliper (Mitutoyo, Kawasaki, Japan). The data are expressed as percentage increase in footpad width (mm). This value is determined by dividing the footpad thickness at time-points 3, 12, 24, and 48 h following subcutaneous (s.c.) injection by the footpad width at Time 0 prior to injection and multiplying by 100. To control for measurement variation, the width of the nonexperimental footpad was monitored throughout the duration of the experiment. The nonexperimental footpad size did not vary significantly from the 48-h time-course. As a negative control, naïve WT mice were s.c.-challenged with α AI, and hind footpad thickness at 3 and 24 h following challenge was measured as described above. No significant increase in footpad width was observed at 3 and 24 h following s.c. α AI challenge of naïve WT mice (results not shown). The percent increase in footpad width following s.c. α AI challenge of naïve WT mice was comparable with that observed in s.c. α AI-challenged, pea-fed mice. In some experiments, the footpad edema is expressed as the percentage increase in footpad width (mm)/CTR. This value is determined by calculating the footpad width in each individual mouse as described above. The value obtained for the experimental group is then expressed as a percentage change in footpad width compared with the appropriate control (antibody control or gene-KO control). For histological analysis of cellular infiltrate, mice were killed 3, 24, and 48 h following antigen challenge, and whole footpads were excised and fixed in formalin for 24 h. The footpad soft tissue was then removed from the foot using a scalpel.

The tissues were fixed, processed, and stained with Charbol's chromotrope-hematoxylin for identification of eosinophils or chloroacetate esterase for the identification of mast cells. The numbers of mast cells and eosinophils in the s.c. region were identified by morphological criteria and quantified per footpad. To quantitate cell levels, the region of the footpad where the s.c. injection was performed was identified ($\times 40$ magnification). The cellular infiltrate was confined between the muscle layer and dermis at the injection site. The number of eosinophils within the complete cellular infiltrate was quantitated. For electron microscopy analysis of inflammatory cell infiltrate, footpad tissue was fixed at 3 and 24 h in 2% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4, overnight at 4°C. Areas of 204 mm² were excised from the footpad and put in 0.1 M Na cacodylate buffer, pH 7.4. Tissue was post-fixed in 1% osmium tetroxide in cacodylate buffer for 1 h and dehydrated in acetone. After dehydration, tissue was infiltrated with Spurr's resin. Sections were cut, mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi H7000 transmission electron microscope.

Serum transfer and CD4⁺ T cell depletion studies

For serum transfer experiments, serum from mice (n=3-4), i.g.-administered with pea or pea- α AI, was transferred intravenously (i.v.) into naïve BALB/c mice (100 μ L/mouse). Two hours following the i.v. injection, hind footpads were challenged with 50 μ L α AI dissolved in PBS (1 mg/mL), or PBS and hind footpad thickness were measured at 0, 3, and 24 h following challenge using a digmatic caliper. For CD4⁺ T cell depletion studies, mice were i.g.-administered pea or pea- α AI plant homogenates as described above. Following the feeding regime, mice received three intraperitoneal (i.p.) injections of α CD4 (0.5 mg/200 μ L; Clone GK1.5) or an isotype control antibody (0.5 mg/200 μ L; Clone BGL113), at 48-h intervals. Following the final i.p. injection, hind footpads were challenged with 50 μ L α AI (1 mg/mL) or PBS, and hind footpad thickness was measured at 0, 3, and 24 h following challenge using a digmatic caliper. To demonstrate that administration of anti-CD4 (α CD4) murine antibody depleted CD4⁺ T cells, spleens were removed from mice at the completion of experimentation, and the presence of CD4⁺ T cells was determined by fluorescein-activated cell sorter. Briefly, spleenocytes were incubated with α CD4-allophycocyanin (Caltag Laboratories, Burlingame, CA; Clone RM4-5) and analyzed by flow cytometry using a FACSVantage SE cell sorter (BD Biosciences, San Jose, CA).

Determination of α AI-specific IgG₁ and IgE in serum

Antibody titers were determined as described previously [15]. In brief, whole blood samples from pea- and pea- α AI-fed mice were taken and microcentrifuged at 13,000 revolutions per minute for 5 min at room temperature. Serum was extracted and stored at -70°C until analysis. α AI-specific IgG₁ levels were determined using an enzyme-linked immunosorbent assay (ELISA). Nunc Maxisorb ELISA 96-well plates were coated with α AI (100 μ g/mL) and incubated overnight at 4°C. The plates were then washed with 0.05% Tween-20 in PBS, blocked with 10% fetal calf serum (FCS) in PBS, and incubated at 37°C for 2 h. Following blocking, plates were washed with 0.05% Tween-20 in PBS, and 100 μ L serum samples were added. Serum samples were diluted 1/10 in 10% FCS. After a 2-h incubation at 37°C, plates were washed with 0.05% Tween-20 in PBS and incubated for 1 h at 37°C with 100 μ L biotin-conjugated anti-mouse IgG₁ (goat anti-mouse IgG₁-biotinylated, Southern Biotechnology Associates, Birmingham, AL; 0.5 μ g/mL). Plates were again washed with 0.05% Tween-20 in PBS, and serum antibody levels were detected using a streptavidin-horseradish peroxidase (HRP) detection system (100 μ L; 2 μ g/mL). The plates were then developed with 100 μ L PharMingen substrate kit (PharMingen, San Diego, CA), and the reaction stopped with 2 N H₂SO₄. Optical densities (OD) were read at 450-570 nm using a 96-well MR-600 microplate reader (Molecular Devices, Sunnyvale, CA). Data are expressed as mean OD at the stated serum dilution. For total and α AI-specific IgE determinations, Nunc Maxisorb ELISA 96-well plates were coated with goat anti-mouse IgE (Southern Biotechnology Associates; 2.0 μ g/mL). Serum samples were diluted 1/10, 100, and 1000 in 10% FCS. Serum total and α AI-specific IgE levels were detected with 100 μ L biotin-conjugated anti-mouse IgE (goat anti-mouse IgE-biotinylated, Southern Biotechnology Associates; 2.0 μ g/mL) or biotin-conjugated α AI (1:250 dilution) with the streptavidin-HRP detection system as described above.

Statistical analysis

The significance of differences between experimental groups was analyzed using Student's unpaired *t*-test. Values are reported as the mean $\pm$ SEM. Differences in means were considered significant if $P < 0.05$.

RESULTS

We have previously demonstrated that the oral consumption of pea- α AI predisposes mice to food allergen-induced cutaneous reactions [13]. To elucidate whether this reaction possessed an early- and late-phase reaction, mice were fed pea or pea- α AI for 4 consecutive weeks and subsequently challenged with purified food allergen α AI by s.c.-injection into the footpad, and the degree of footpad edema was measured at 3, 12, 24, and 48 h (Fig. 1). Footpad width in mice fed pea- α AI was significantly increased as compared with those fed the pea at 3, 12, and 24 h following antigen challenge and declined after 24 h to return to baseline at 48 h (Fig. 1).

Histological examination of footpad biopsy specimens from pea- α AI-fed mice 3 h following α AI s.c. challenge revealed mild epidermal spongiosis and inflammatory cells in the dermis, which included eosinophils and mast cells (Fig. 2). Twenty-four hours following α AI s.c. challenge, acanthosis and a pronounced cellular infiltrate of the dermis were observed in pea- α AI-fed mice (Fig. 2, b and d) but not in pea-fed mice (Fig. 2, a and c). The inflammatory infiltrate primarily consisted of eosinophils; however, mast cells and mononuclear cells were also observed. Quantification of inflammatory cells in the footpad biopsy specimens 3 h following s.c. α AI challenge revealed a significant increase in eosinophil accumulation in the footpad in pea- α AI-fed mice as compared with α AI s.c.-challenged pea-fed mice (Fig. 2e). By 24 h, eosinophil numbers had increased by 3.5-fold in pea- α AI-fed mice before returning to baseline levels comparable with that observed in pea-fed mice (48 h). In contrast, we observed no change in mast cell numbers in the footpad of pea- α AI-fed mice at 3 h as

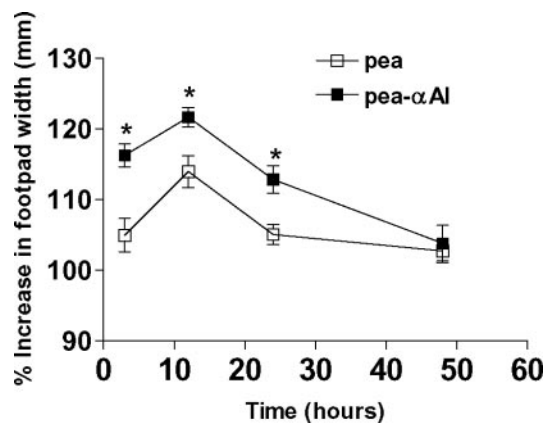


Fig. 1. Time-course of experimental food allergen-induced cutaneous allergic reaction. Percentage (%) increase in footpad size 3, 12, 24, and 48 h following α AI s.c. challenge in pea- and pea- α AI-fed mice. Data expressed at the mean percent change in footpad size $\pm$ SEM from $n = 4$ –6 mice per group from triplicate experiments. Statistical significance of difference was determined using Student's unpaired *t*-test. *, P value < 0.05 .

compared with α AI s.c.-challenged pea-fed mice (Fig. 2f). It is interesting that 24 h following α AI challenge, a sharp decline in the number of mast cells in the footpad of pea- α AI-fed mice, when compared with pea-fed α AI-challenged mice, was observed (Fig. 2f). Forty-eight hours following α AI challenge, mast cell numbers returned to levels comparable with that observed in pea-fed mice.

To examine the activation status of the mast cells and eosinophils in the footpad, we performed electron microscopy analysis of biopsy samples 3 and 48 h following s.c. α AI challenge. Mast cells in biopsy samples from pea-fed mice 3 h following s.c. challenge with α AI were intact with typical, round, homogeneous electron dense secretory granules (Fig. 3a), only few of which showed signs of degranulation. By contrast, mast cells in the footpad of s.c. α AI-challenged pea- α AI-fed mice demonstrated extensive degranulation with obvious signs of exocytosis, which is commonly seen during anaphylaxis (Fig. 3, b and c). Eosinophils in biopsy samples 24 h following s.c. α AI-challenge of pea-fed mice were located primarily in the dermis and possessed features including intact plasma membranes, heterochromatic-segmented nuclei, and abundant granules with characteristic electron dense cores and matrixes (Fig. 3d). By comparison, eosinophils in s.c. α AI-challenged pea- α AI-fed mice appeared to be undergoing cytolytic eosinophilic degranulation, as evidenced by nuclear chromatolysis, disruption of plasma membrane, and the presence of free eosinophilic granules in the extracellular spaces adjacent to these eosinophils (Fig. 3, e and f). Eosinophils in α AI-challenged pea- α AI-fed mice 3 h following α AI challenge were not undergoing degranulation and appeared similar to that observed in pea-fed mice (results not shown; Fig. 3d). Collectively, these studies demonstrate that experimental food allergen-induced cutaneous reactions consist of an ECR and LCR and that mast cells are involved in the early-phase and eosinophils in the late-phase eczematous reaction.

It is postulated that Ig, particularly IgE and mast cells, are important in the early-phase component of the cutaneous reaction [16]. Furthermore, the LCR is thought to be dependent on the mast cell-mediated, allergic, inflammatory ECR. First, to examine whether oral consumption of pea- α AI elicited an Ig response, we examined total IgE levels in mice, which were orally administered pea or pea- α AI seed meal. Levels of total IgE in the mice that were fed pea- α AI were significantly higher than that observed in pea-fed mice [total IgE in serum 1/10 dilution: 0.46 ± 0.02 vs. 0.26 ± 0.03 , $P < 0.001$; pea- α AI vs. pea-fed mice, respectively; data represent mean $\pm$ SD OD (450 nm); $n = 4$ and 6 mice per group]. To determine whether oral consumption of pea- α AI elicited an α AI-specific Ig response, we examined α AI serum antibody titers in these mice [Fig. 4a (i and ii)]. It is notable that α AI-specific IgG₁ and IgE were detected at significant levels in mice, which were fed pea- α AI but not pea [Fig. 4a (i and ii)]. To examine the contribution of Ig in food allergen-induced ECR and the role of this pathway in the induction of the LCR, serum from mice fed the pea or pea- α AI was transferred into naïve mice, and footpad edema was measured at 3 and 24 h following s.c.-challenge of α AI. Mice receiving serum from animals fed the pea- α AI and challenged in the footpad with α AI have a significant increase in footpad swelling at 3 h but not at 24 h (Fig. 4b) following

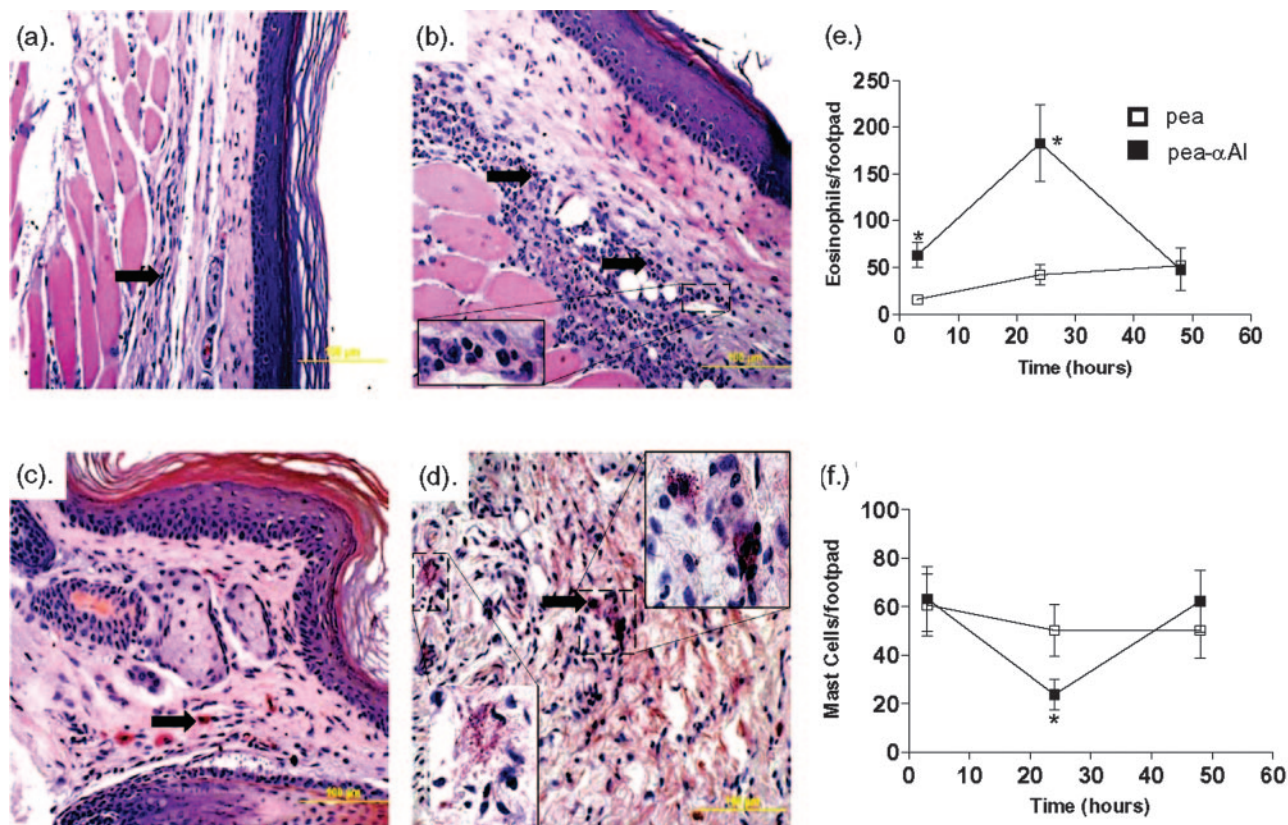


Fig. 2. Mast cell and eosinophil involvement in experimental food allergen-induced allergic-cutaneous reaction. Representative photomicrographs of chromotrope (a and c)- and chloroacetate esterase (b and d)-stained footpad sections from pea-fed (a and c) or pea-αAI-fed (b and d) mice s.c.-challenged with αAI. Quantification of eosinophils (e) and mast cell numbers (f) in the footpad of pea- and pea-αAI-fed mice s.c.-challenged with αAI. Data are the mean eosinophils/footpad (b) or mast cells/footpad (c) ± SEM from $n = 4-6$ mice per group from duplicate experiments. Statistical significance of difference was determined using Student's unpaired *t*-test. *, *P* value <0.05. Representative photomicrographs taken at original magnification ×40 and ×100 in insets. Black arrows indicate eosinophils (a and b) or mast cells (c and d).

antigen challenge, when compared with those receiving serum from pea-fed mice. These studies demonstrate that the early phase of the hypersensitivity response (3 h post-antigen challenge) was mediated by Ig. Furthermore, these studies show that the ECR alone is not sufficient for the induction of the LCR.

During the food allergen-induced LCR, we observed a cellular infiltrate in the skin consisting of mononuclear cells and eosinophils. Immunohistochemical analysis of the mononuclear cell infiltrate in a LCR in atopic subjects has revealed the majority of the mononuclear cell population to be CD4⁺ T cells [17]. Furthermore, increased levels of CD4⁺ T cells have been shown to correlate with eosinophil accumulation [18]. To examine the role of CD4⁺ T cells in experimental food allergen-induced LCR, mice were fed pea-αAI and subsequently administered αCD4 neutralizing monoclonal antibody to deplete CD4⁺ T cells (GK 1.5) or control Ig (βGL113) and challenged with αAI s.c. in footpad. αAI challenge of control Ig-treated pea-αAI-fed mice induced footpad swelling (Fig. 4c). In contrast, αAI challenge of αCD4-treated pea-αAI-fed mice did not induce a late-phase footpad swelling response (Fig. 4c). No difference in early-phase footpad edema was observed between αCD4- and control Ig-treated, pea-αAI-fed mice 3 h following s.c. challenge with αAI (results not shown). To examine whether the reduction in footpad edema by αCD4

treatment was associated with decreased eosinophil levels, we quantitated eosinophils in the footpad of these mice. αCD4-treated pea-αAI-fed mice s.c.-challenged with αAI had significantly lower levels of eosinophils in the footpad than Ig control-treated, pea-αAI-fed mice s.c.-challenged with αAI (Fig. 4d). The eosinophil levels in the footpad of these mice were equivalent to that observed in Ig control-treated, pea-fed mice s.c.-challenged with αAI. These results suggest that CD4⁺ T cells regulate the influx of eosinophils in food antigen-induced LCR.

CD4⁺ T cells are thought to regulate eosinophil recruitment to the site of allergic skin reaction through the activity of Th₂ cytokines [19–22]. To examine the role of these molecules in food allergen-induced LCR, IL-4Rα^{-/-}, IL-13^{-/-}, and eotaxin-1^{-/-} mice were fed pea or pea-αAI seed meal and subsequently s.c.-injected with αAI, and footpad edema and eosinophil infiltration into the footpad were examined. s.c. challenge of IL-4Rα^{-/-}, IL-13^{-/-}, and eotaxin-1^{-/-} mice fed pea-αAI did not induce an ECR (3 h) or LCR (24 h), as measured by an increase in footpad width, when compared with pea-αAI-fed WT mice (Fig. 5, a and b). Consistent with this observation, no significant increase in the number of eosinophils in the cellular infiltrate in the injected footpads of IL-4Rα^{-/-}, IL-13^{-/-}, and eotaxin-1^{-/-} pea-αAI-fed mice was observed in comparison with controls (Fig. 5c). These findings

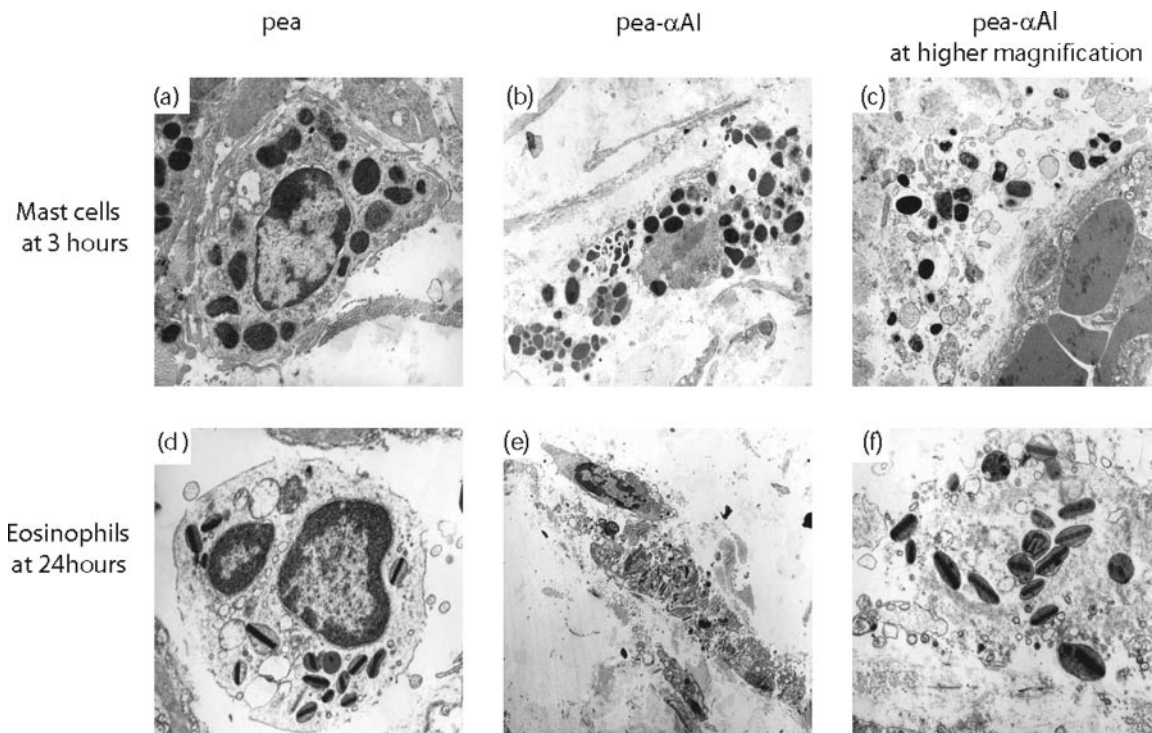


Fig. 3. Mast cell and eosinophil degranulation during experimental food allergen-induced ECR and LCR. Representative electron photomicrographs of mast cells (a–c) or eosinophils (d–f) in the footpad dermis of pea (a and d)- and pea- α AI (b, c, e, and f)-fed mice s.c.-challenged with α AI. Original magnification of representative photomicrographs is 8000 $\times$ (a), 5000 $\times$ (b), 7000 $\times$ (c), 12,000 $\times$ (d), 5000 $\times$ (e), and 17,000 $\times$ (f).

suggest that IL-13, IL-4R α chain, and eotaxin-1 play important roles in eosinophil recruitment into allergic skin and that eosinophils appear to be responsible for the observable edema and inflammation during food allergen-induced LCR.

DISCUSSION

There has been extensive debate concerning the contribution of food in the onset and exacerbation of cutaneous allergic diseases including AD. We have recently developed an adjuvant-independent, experimental model of food allergy, which predisposes mice to cutaneous allergic reactions [13]. In the present study, we have extended these initial observations and have dissected out the key cells and molecules involved in food allergen-induced, allergic cutaneous reactions in this experimental system. We show that food allergen-induced cutaneous reactions consisted of an ECR and a LCR; the ECR was associated with mast cell degranulation and dependent on Ig, the LCR was CD4⁺ T cell-dependent and associated with an eosinophilic infiltrate, and the LCR was critically regulated by pathways that use the eotaxin-1, IL-13, and IL-4R α chain.

Clinical investigations have demonstrated that skin contact with an allergic food can induce a variety of cutaneous manifestations including contact urticaria, allergic contact dermatitis, and protein contact dermatitis [5–7, 23]. It is notable that food handlers develop a protein-contact dermatitis within 30 min following skin contact with allergic food groups including raw seafood, meats, vegetables, fruits, and spices [24]. It is postulated that the underlying mechanism of this reaction is a

combination of immediate (Type 1) and delayed (Type 4) hypersensitivity reactions [25]. In the present study, we demonstrate that exposure of the skin of food-allergic mice to food allergen can promote a biphasic hypersensitivity response characterized by an early-phase, skin-swelling reaction that peaks 2–3 h following challenge (Type 1 reaction), followed by a late-phase, cell-mediated response (Type 4 reaction) 24–48 h after allergen challenge.

The ECR component is thought to be mediated by a mast cell/IgE-induced increase in vascular permeability [16, 26]. Mast cells following antigen/IgE activation release an array of mediators such as leukotriene B₄ (LTB₄), LTC₄, LTD₄, prostaglandin D₂ (PGD₂), serotonin, histamine, and chemokines, promoting vascular permeability and local edema. Consistent with this hypothesis, we demonstrate that transfer of serum containing α AI-specific IgE was sufficient to induce ECR. Furthermore, we show that food allergen-induced ECR was associated with mast cell degranulation. It is notable that we observed a decrease in mast cell numbers at 24 h following α AI challenge. We speculate that this was a result of loss of histological detection by chloroacetate esterase staining following mast cell degranulation at 3 h.

Clinical and experimental investigations suggest that early-phase cutaneous mast cell degranulation also plays a role in the augmentation of the LCR [22, 27–31]. The mast cell-derived mediators (LTB₄, LTC₄, LTD₄, PGD₂, and chemokines) are thought to promote vascular permeability and the attraction of CD4⁺ T cells and eosinophils into the site of inflammation [29, 32, 33]. Serum transfer studies revealed that the ECR reaction was not sufficient to induce a LCR. Currently, we

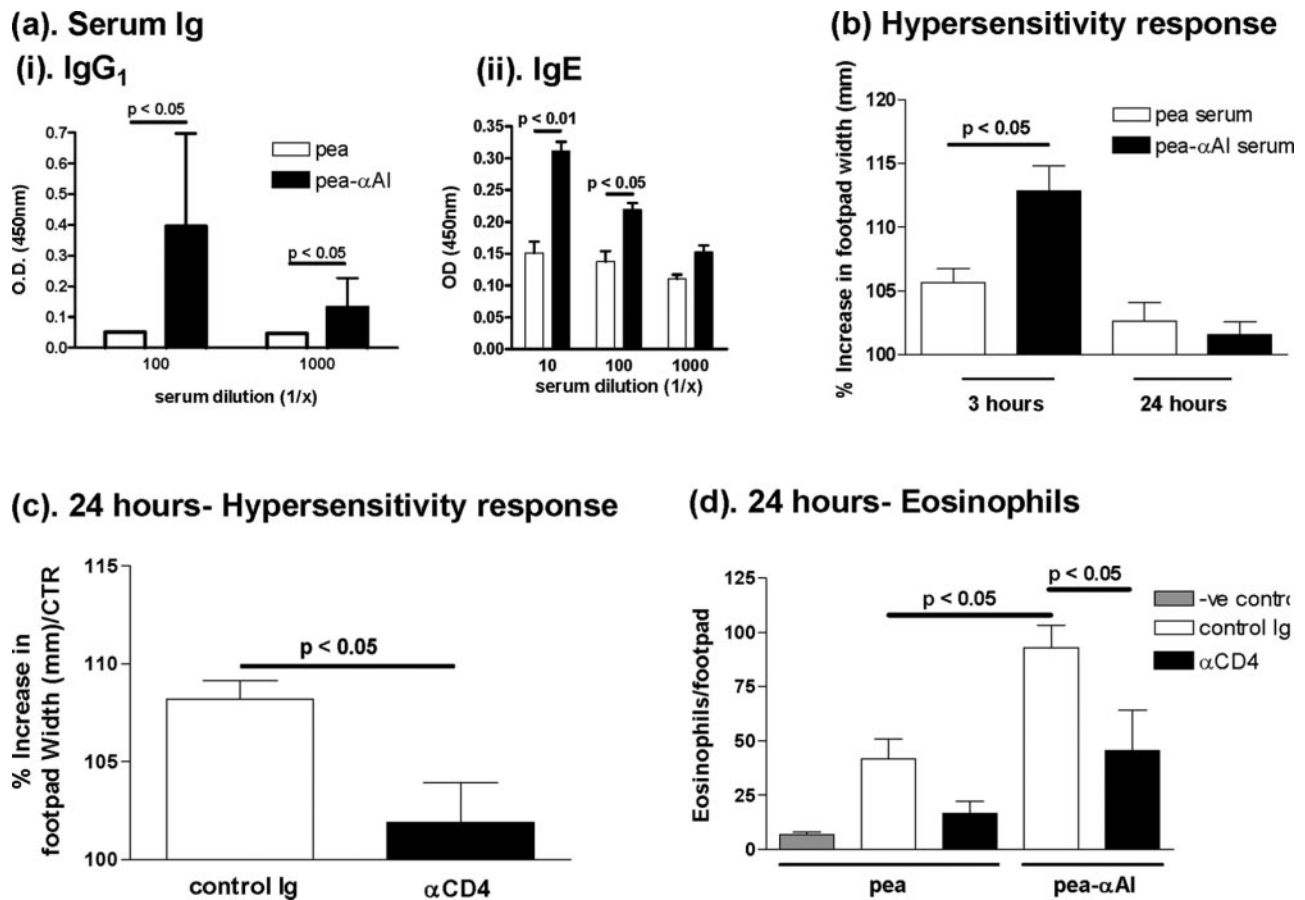


Fig. 4. Experimental food allergen-induced cutaneous allergic reaction is mediated by serum factors and CD4⁺ T cells. (a) Antigen-specific (i) IgG₁ and (ii) IgE in serum of pea- and pea- α AI-fed mice. (b) Percent increase in footpad width in naïve mice receiving serum from pea- or pea- α AI-fed mice and s.c.-challenged with α AI. (c) Percent increase in footpad width/CTR in pea- α AI-fed mice treated with α CD4 or control Ig antibody and s.c.-challenged with α AI. (d) Eosinophil numbers in the footpad of pea- α AI-fed mice treated with α CD4 or control Ig antibody and s.c.-challenged with α AI. –ve control, Eosinophils/footpad of pea-fed mice 24 h following s.c. challenge with vehicle. (c) Data expressed as the mean percent change in footpad size in pea- α AI-fed mice treated with α CD4 or control Ig antibody and s.c.-challenged with α AI as compared with the respective control (pea-fed mice treated with α CD4 or control Ig antibody and s.c.-challenged with α AI). (a) Mean OD of the serum dilution $\pm$ SD from four to six mice per group and (b–d) mean $\pm$ SEM at 3 or 24 h following s.c. challenge of α AI, from $n = 4$ –6 mice per group from duplicate experiments. Statistical significance of differences ($P < 0.05$) was determined using Student's unpaired t -test.

cannot fully account for these differences; however, a number of clinical studies have been unable to demonstrate a correlation between specific IgE and clinical response to food in food-responsive AD, suggesting IgE/mast cell-independent mechanisms in the pathogenesis of food allergen-induced AD [34, 35]. It is possible that food allergen-induced sensitization and challenge activates both the mast cell/IgE and CD4⁺ Th₂/eosinophil pathways simultaneously but they function as two independent, inflammatory cascades. Thus, transfer of one component of the response, in our case, serum and not CD4⁺ T cells would be insufficient to reconstitute the ECR and LCR.

The food allergen-induced LCR cellular infiltrate was primarily comprised of lymphocytes and eosinophils. Eosinophil infiltration into the site of inflammation is thought to involve Th₂ cytokine and chemokine pathways [36]. We further demonstrate that eosinophil infiltration into the skin during LCR was dependent on IL-13, IL-4R α , and eotaxin-1. IL-13 is a proinflammatory cytokine produced at high levels by activated CD4⁺ Th₂ cells and is central to the activation of a number of Th₂ pathways including the up-regulation of the eosinophil-selective chemokine, eotaxin-1 expression [37–39]. IL-13

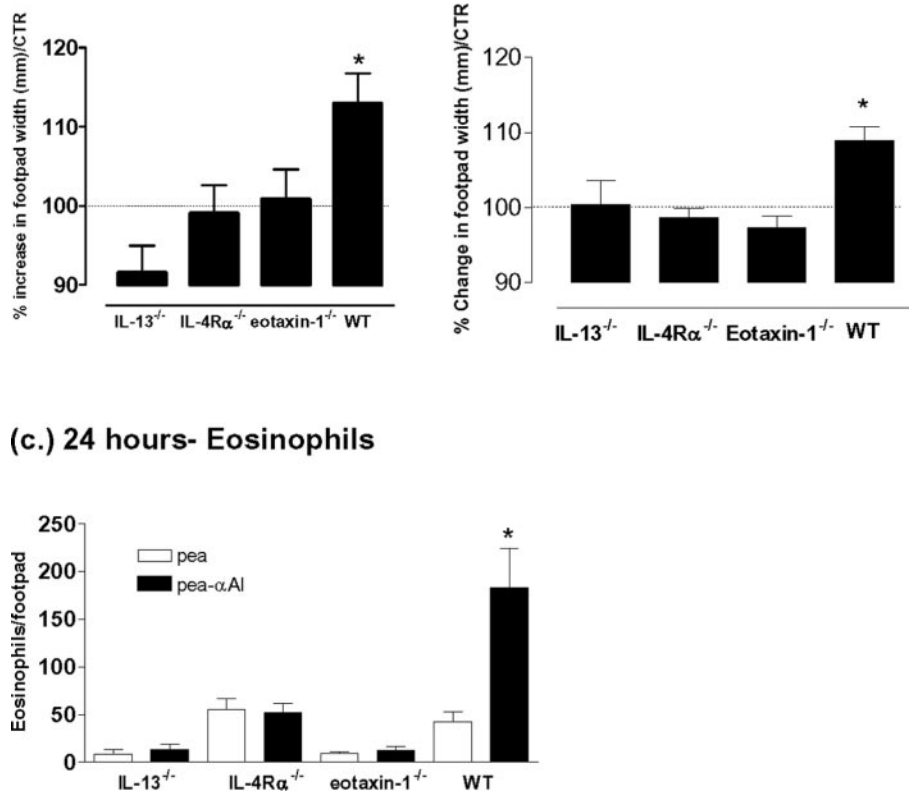
mRNA levels have been found to be up-regulated in contact hypersensitivity [40] and in dermal fibroblasts [41]. IL-13 signals through two receptors: IL-13R Type 1 (IL-13R α ₁–IL-13R α ₂ subchains) and IL-4R Type 2 (IL-4R α –IL-13R α ₁ chains). We show that IL-13-mediated eosinophil recruitment in classical LCR is dependent on the IL-4R α chain, suggesting IL-13-mediated effects are mediated predominantly through IL-4R Type 2. Eotaxin-1 is the most potent and selective eosinophil chemoattractant and has been shown to be important in eosinophil trafficking during allergic inflammatory responses, including to the site of allergic skin reactions in humans [22, 36, 42, 43]. Eotaxin-1 primarily signals through the CC chemokine receptor 3 (CCR3), and experimental investigations have shown eosinophils to constitutively express CCR3 [44]. Indeed, CCR3 and eotaxin-1 expression is up-regulated in lesional skin from AD as compared with controls [45]. Furthermore, intradermal (i.d.) injection of eotaxin-1 in humans induces an eosinophilic infiltrate [46].

An alternate explanation for the observed, attenuated ECR and LCR responses in IL-13^{–/–}, IL-4R α ^{–/–}, and eotaxin-1^{–/–} mice is that these pathways play an important role in the

(a.) 3 hours

(b.) 24 hours

Fig. 5. Experimental food allergen-induced ECR and LCR are dependent on IL-13, IL-4R α chain, and eotaxin-1. Percent increase in footpad width (mm)/CTR in pea- α AI-fed IL-13 $^{-/-}$, IL-4R α chain $^{-/-}$, and eotaxin-1 $^{-/-}$ and WT mice 3 h (a) and 24 h (b) following s.c. challenge with α AI. (c) Eosinophil numbers in the footpad of pea- α AI-fed IL-13 $^{-/-}$, IL-4R α chain $^{-/-}$, and eotaxin-1 $^{-/-}$ and WT mice s.c.-challenged with α AI. (a and b) Data expressed as the mean percent change in footpad size in pea- α AI-fed IL-13 $^{-/-}$, IL-4R α chain $^{-/-}$, and eotaxin-1 $^{-/-}$ and WT mice compared with the respective control (pea-fed IL-13 $^{-/-}$, IL-4R α chain $^{-/-}$, and eotaxin-1 $^{-/-}$ and WT mice s.c.-challenged with α AI). (c) Mean eosinophil numbers/high-power field $\pm$ SEM, 24 h following α AI or vehicle challenge, from $n = 4$ –6 mice per group from duplicate experiments. Statistical significance of differences ($P < 0.05$) was determined using Student's unpaired t -test. *, P value < 0.05 .



induction phase of sensitization of the food (α AI). To confirm α AI sensitization in these mice, we examined the presence of α AI-specific Ig in the serum of pea- and pea- α AI-fed IL-13 $^{-/-}$, IL-4R α $^{-/-}$, and eotaxin-1 $^{-/-}$ mice. We detected α AI-specific IgG $_1$ in the serum of pea- α AI-fed WT and eotaxin-1 $^{-/-}$ mice [α AI-specific IgG $_1$ in serum, 1/100 dilution: 0.09 ± 0.03 vs. 0.25 ± 0.07 , $P < 0.05$; pea- vs. pea- α AI-fed eotaxin-1 $^{-/-}$ mice, respectively; data represent mean $\pm$ SD OD (450 nm); $n = 4$ mice per group]; however, we were unable to detect any α AI-specific IgG $_1$ in pea- α AI-fed IL-4R α $^{-/-}$ and IL-13 $^{-/-}$ mice [α AI-specific IgG $_1$ in serum 1/100 dilution: 0.15 ± 0.05 vs. 0.13 ± 0.08 and 0.01 ± 0.01 vs. 0.01 ± 0.01 ; pea- vs. pea- α AI-fed IL-13 and IL-4R α $^{-/-}$ mice, respectively; data represent mean $\pm$ SEM OD (450 nm); $n = 3$ –5 mice per group]. Recently, we have demonstrated that intratracheal (i.t.) challenge of pea- α AI orally sensitized mice with α AI promotes exaggerated α AI-specific IgG $_1$ and Th $_2$ pulmonary inflammation [13]. To test whether α AI sensitization had occurred in IL-13 $^{-/-}$ and IL-4R α $^{-/-}$ mice, pea- and pea- α AI-fed IL-4R α $^{-/-}$ and IL-13 $^{-/-}$ mice were i.t.-challenged with α AI and α AI-specific IgG $_1$ and evidence of Th $_2$ pulmonary inflammation was examined [13]. Oral administration of pea- α AI to WT and IL-13 $^{-/-}$ mice and subsequent i.t. administration of α AI induced detectable levels of α AI-specific IgG $_1$ and Th $_2$ pulmonary inflammation, confirming α AI sensitization (results not shown). However, we did not detect any α AI-specific IgG $_1$ or evidence of Th $_2$ pulmonary inflammation in IL-4R α $^{-/-}$ mice. These studies demonstrate α AI sensitization in WT, IL-13 $^{-/-}$, and eotaxin-1 $^{-/-}$ mice, suggesting that IL-13 and eotaxin-1 regulate the LCR response, probably through modulation of

eosinophil recruitment and effector function, whereas the IL-4R α pathway may play a role in oral antigen sensitization.

Recent studies using an adjuvant-based s.c. hypersensitivity model have demonstrated that eosinophil infiltration during delayed-type hypersensitivity (DTH) responses is attenuated in CXC chemokine receptor 3 (CXCR3)-deficient mice [21]. In our food allergen-induced model system, we demonstrate that eosinophil recruitment was dependent on eotaxin-1, a CCR3-sensitive pathway. We attribute these differences to several possibilities, including the use of an adjuvant (complete Freund's adjuvant) model system and that ablation of the CXCR3 pathway by genetic modification could attenuate the T cell infiltration during the DTH response and thus, inhibit expression of eosinophil-specific signals for eosinophil recruitment. Indeed, we have demonstrated that the LCR is CD4 $^+$ T cell-dependent, and CXCR3 has been shown to be expressed on T cells and play an important role in T cell activation [47–50].

The attenuation of the LCR in eotaxin-1- and IL-13-deficient mice and the association with eosinophils suggest that this leukocyte plays an important role in the mediation of food allergen-induced cutaneous reactions. Indeed, our electron microscopy studies revealed extensive eosinophil degranulation following. The presence of extracellular eosinophil granule proteins deposited in the skin has often been observed in eosinophil-associated, cutaneous disorders such as AD [2, 51–53]. Furthermore, i.d. administration of eosinophil granule proteins has also been shown to promote cutaneous vasopermeability [54].

We have elucidated that food allergen-induced cutaneous allergic reactions are associated with CD4⁺ Th₂ immune responses and are critically regulated by the IL-13/IL-4R α chain pathway. It is tempting to speculate upon skin contact with food allergen, food allergen-reactive CD4⁺ Th₂ cells translocate into the site of allergen challenge and orchestrate the allergic inflammatory response. Indeed, in vitro stimulation of T cells and T cell clones from food-allergic patients produce Th₂ cytokines (IL-4, IL-5, and IL-13) following antigen stimulation [55–58]. Furthermore, in support of this hypothesis, food antigen-reactive T cells from patients with AD and urticaria consist of a greater number of CD4⁺ cutaneous lymphocyte antigen (CLA)⁺ T cells as compared with normal individuals [59, 60]. The CLA is the unique skin-homing receptor that binds E-selectin, which is constitutively expressed on dermal microvasculature, promoting lymphocyte trafficking to skin [61, 62].

Unlike previously developed food-allergy models, our model is mediated by adjuvant-independent processes. This adjuvant-independent food antigen model has provided us with a unique opportunity to elucidate the natural cellular and molecular mechanisms involved in food allergen-induced cutaneous allergic reactions. These studies demonstrate that food-allergen sensitization can predispose to an allergic ECR and LCR. Furthermore, we show that the ECR is regulated by mast cells and Ig-dependent pathways and LCR, by eotaxin-1-, IL-4R α -, and IL-13-dependent mechanisms. Collectively, these studies highlight that food allergy may predispose to cutaneous allergic reactions.

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